RESEARCH ARTICLE

In vivo X-ray diffraction and simultaneous EMG reveal the time course of myofilament lattice dilation and filament stretch

Sage A. Malingen1,*, Anthony M. Asencio1, Julie A. Cass2, Weikang Ma3, Thomas C. Irving3 and Thomas L. Daniel1,*

ABSTRACT

Muscle function within an organism depends on the feedback between molecular and meter-scale processes. Although the motions of muscle’s contractile machinery are well described in isolated preparations, only a handful of experiments have documented the kinematics of the lattice occurring when multi-scale interactions are fully intact. We used time-resolved X-ray diffraction to record the kinematics of the myofilament lattice within a normal operating context: the tethered flight of Manduca sexta. As the primary flight muscles of M. sexta are synchronous, we used these results to reveal the timing of in vivo cross-bridge recruitment, which occurred 24 ms (s.d. 26) following activation. In addition, the thick filaments stretched an average of 0.75% (s.d. 0.32) and thin filaments stretched 1.11% (s.d. 0.65). In contrast to other in vivo preparations, lattice spacing changed an average of 2.72% (s.d. 1.47). Lattice dilation of this magnitude significantly affects shortening velocity and force generation, and filament stretching tunes force generation. While the kinematics were consistent within individual trials, there was extensive variation between trials. Using a mechanism-free machine learning model we searched for patterns within and across trials. Although lattice kinematics were predictable within trials, the model could not create predictions across trials. This indicates that the variability we see across trials may be explained by latent variables occurring in this naturally functioning system. The diverse kinematic combinations we documented mirror muscle’s adaptability and may facilitate its robust function in unpredictable conditions.

KEY WORDS: Myofilament lattice dynamics, Sarcomere, Structure–function

INTRODUCTION

Using ubiquitous molecular machinery, muscle performs diverse functions within an organism: functioning as a motor, structural support, a repository for elastic energy, or as a shock absorber (Dickinson et al., 2000). A muscle’s functional output—the force it creates and its length change—depends upon multi-scale interactions. Arrays of molecular motors interact (generating piconewton-scale forces) in a feedback loop with interacting muscle groups (generating newton-scale forces) within the animal’s body. The highly organized lattice of contractile machinery that powers contraction also changes shape as a result of internal forces, temperature and externally applied forces. In turn, the shape of the lattice tunes force production.

For instance, increasing the spacing between molecular motors and thin filaments to which they bind decreases binding probability, and therefore decreases force output (Williams et al., 2013). The spacing of the lattice co-varies with naturally occurring temperature gradients, and has the potential to shift the function of otherwise identical muscle sub-units from motors to springs and dampers (George et al., 2012, 2013). Additionally, the thick and thin filaments that house myosin molecular motors and actin binding sites are far from a rigid system; instead they stretch in conjunction with internal axial forces and muscle activation. Filament stretching has important mechanical implications, accounting for about 70% of a muscle’s total compliance (Wakabayashi et al., 1994), and resulting in increased binding probability due to changes in the axial register of molecular motors and prospective binding sites (Daniel et al., 1998). Although lattice dilation (Metzger and Moss, 1987; Fukuda et al., 2005; Williams et al., 2013) and filament stretch (Squire, 1997) have noteworthy mechanical implications, the relationship of their magnitude and timing within a naturally functioning organism has remained enigmatic. To address this, we documented molecular motor recruitment following nervous activation, filament stretching and changes in lattice spacing in a novel in vivo experimental system: the synchronous flight muscles of the hawkmoth Manduca sexta.

The advent of high-speed digital detectors in the early 2000s made in vivo, time-resolved X-ray diffraction possible. Landmark studies of lattice kinematics in naturally functioning systems have largely focused on asynchronous insect flight muscles (fruitfly: Drosophila and bumblebee: Bombus) (Irving and Maughan, 2000; Dickinson et al., 2005; Iwamoto and Yagi, 2013). Irving and Maughan, for example, were the first to document the molecular kinematics of a mutant fly in vivo, connecting molecular mutations with their functional outcomes across the scales of animal flight behavior (Irving and Maughan, 2000). However, one limitation of these systems is that in asynchronous muscle neural activation is decoupled from cycles of muscle shortening and lengthening; instead activation serves to keep the muscle in a contractile state by continuously suffusing myofibrils with calcium ions. Asynchronous muscle is uniquely specialized to power high-frequency wing flapping (Syme and Josephson, 2002). Iwamoto and Yagi leveraged this to record the mechanism of stretch activation independent of calcium release and re-uptake (Iwamoto and Yagi, 2013).

In contrast to asynchronous muscle, many mammalian skeletal muscles are activated in partial tetany, with multiple neural impulses stimulating larger contractile forces. Vertebrate cardiac muscle, however, has a one-to-one relationship between activation and contraction. In a manner analogous to cardiac muscle, the dominant flight muscles of M. sexta are generally synchronous with a one-to-one...
Table 1. For each of the 11 total trials and each data type a permutation test was run to see if there was a significant frequency component at wing beat frequency

<table>
<thead>
<tr>
<th>Data type</th>
<th>No. of trials</th>
<th>Baseline (nm)</th>
<th>Excursion (nm)</th>
<th>Excursion (%)</th>
<th>Peak time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i2.0/i1.0</td>
<td>9</td>
<td>0.70±0.076</td>
<td>0.25±0.204</td>
<td>35.3±26.92</td>
<td>0.024±0.026</td>
</tr>
<tr>
<td>7.3 mm centroid</td>
<td>7</td>
<td>7.29±0.02</td>
<td>0.06±0.02</td>
<td>0.75±0.32</td>
<td>0.058±0.024</td>
</tr>
<tr>
<td>5.9 mm centroid</td>
<td>6</td>
<td>5.87±0.04</td>
<td>0.07±0.04</td>
<td>1.11±0.65</td>
<td>0.044±0.025</td>
</tr>
<tr>
<td>d1.0</td>
<td>11</td>
<td>45.84±0.88</td>
<td>1.24±0.66</td>
<td>2.72±1.47</td>
<td>0.032±0.018</td>
</tr>
<tr>
<td>14.3 centroid</td>
<td>6</td>
<td>14.26±0.08</td>
<td>0.30±0.13</td>
<td>2.08±0.91</td>
<td>0.039±0.026</td>
</tr>
</tbody>
</table>

Values are mean±s.d. If the chance of a random permutation yielding a power as high or higher than the raw data at wing beat frequency was less than 5% we factored it into this summary. If it was greater than 5% we can only conclude that if there are periodic changes occurring, they are of lower amplitude than the noise envelope. The number of trials that passed the permutation test is recorded in the column No. of trials. For each trial and data type, its baseline value was the minimum point in the STA. The average baseline is the mean across all trials. The excursion of each data type was calculated as the average across all trials of the STA maximum minus the STA minimum. The percent excursion was the absolute excursion divided by the baseline for each trial – these were averaged across all trials. Finally, the peak time was the time elapsed from the beginning of the STA to its maximum, which was averaged across all trials.

correspondence between neural activation and muscle contraction. Additionally, they mirror cardiac muscle in their function, contracting against fluid loads and acting predominantly on the ascending portion of their length–tension curve (Tu and Daniel, 2004).

By pairing simultaneous recording of muscle activation with X-ray diffraction data, we were able to phase average lattice kinematics using the organism’s natural activation. These results uniquely show the timing of molecular motor recruitment and the corresponding activation (Table 1). Using phase averaged data we found that the molecular kinematics are consistent within individuals, often showing large excursions: the lattice can dilate by as much as 2.5 nm (5.4%), thick filaments can stretch by as much as 1.2%, and thin filaments by as much as 2.1%. In light of experimental and computational studies, the magnitude of these results support the notion that lattice kinematics significantly modulate muscle function (Metzger and Moss, 1987; Daniel et al., 1998; Williams et al., 2013). As is expected in a fully intact, normally functioning animal, there was considerable variation in both the timing and extent of lattice motions across individuals. These results highlight that while individuals may manifest the same functional outcome at the organism scale (flight), the mechanics of the lattice underneath can vary. Considering this, lattice kinematic data need to be considered within the context of individuals without supposing that latent variables are equal across individuals (Gomez-Marin and Ghazanfar, 2019).

**MATERIALS AND METHODS**

**Animal preparation**

To record cross-bridge recruitment and lattice kinematics as a function of electrical activation, we used an insect synchronous muscle [*Manduca sexta* (Linnaeus 1763)]. The dorsal longitudinal muscles (DLMs) of *M. sexta* are synchronously activated, and are an excellent system for X-ray diffraction experiments as the thorax is composed of about a cubic centimeter of highly ordered muscle and the exoskeleton produces negligible scattering. The clear diffraction patterns produced by this muscle made it possible to collect time resolved data without frame averaging. We used a random mix of male and female moths 1–2 weeks post-eclosion. We did not record sex and our limited sample size precludes analysis of sex differences.

We cold-anesthetized a moth, tethered it and placed it in the beam line (Fig. 1). Moths were unencumbered except by electromyography (EMG) electrodes inserted into the thorax and the ventral tether. The tether was a flattened stainless-steel needle coated in cyanoacrylate glue inserted between the second and third coxae and crystalized with sodium bicarbonate. The moth was positioned on the tether with a pitch angle of approximately 30 deg to the horizon, similar to natural flight orientation. The moth was placed on the beamline with its body axis, and hence the axis of the DLMs, at a right angle to the beam’s incidence. The beam passed through the anterior, dorsal quadrant of the thorax, intersecting at approximately the d-c DLM subgroup based on external morphology (Eaton et al., 1988). It was aimed consistently in a small section where the wings would not obstruct the beam path. A hot air soldering iron set to its lowest heat setting was placed approximately 1 m in front of the moth while a fan with a filter attached was placed behind the moth to collect dislodged scales. The warmth of the soldering iron and the wind current created by it and the fan stimulated natural flight behavior and kept the moth warmer than the room’s cool ambient temperature.

**Electromyogram**

EMG electrodes with hooked tips were inserted into the posterior of the thorax after puncturing the exoskeleton with a needle. The
ground electrode was inserted into the abdomen (Fig. 2). In order to record the rapid electrical transient of muscle activation, the EMG must be recorded at high temporal resolution. Therefore we recorded these data at 25,000 Hz. As the purpose of recording muscle activation was to correlate it with the kinematics measured using X-ray diffraction, we rounded the timing of muscle activation to the nearest 1/200th of a second in order to correspond with the time base we used to record lattice kinematics. Because of this it may appear that there is jitter in the peak detection. The data (both X-ray diffraction and EMG) from animals that did not have periodic EMG signals with identifiable peaks for the full 1 s recording were excluded.

**X-ray diffraction**

**Beamline set-up**

The experiment was performed at the Biophysics Collaborative Access Team (BioCAT) beamline 18ID at the Advanced Photon Source, Argonne National Laboratory, Lemont, IL, USA (Fischetti et al., 2004). The beam energy was 12.0 keV with an incident flux of $10^{13}$ photons s$^{-1}$ and attenuated as needed to $10^{12}$ photons s$^{-1}$. It was focused to $250 \times 250$ µm at the animal’s thorax, and $60 \times 150$ µm at the detector with a sample to detector distance of 2 m. The sample was oscillated over a 1 mm excursion in the beam at 20–30 mm s$^{-1}$ in order to mitigate radiation damage. This method of oscillating the tissue relies upon the supposition that all sarcomeres in a local region are behaving in the same manner, so moving the sample will not alter the X-ray periodicities observed. We used a photon counting Pilatus 3X1M (Dectris Inc., Baden, Switzerland) with 981×1043 and 172×172 µm pixels with a 20-bit dynamic range.

Raw image data were collected at 200 Hz with 1 ms of dead time per frame, and with the X-ray shutter open continuously.

**Data reduction from X-ray diffraction images**

The 32-bit tagged image file format (TIFF) image stacks returned by the X-ray detector were annotated using the Musclex software suite developed by BioCAT (Jiratrakanvong et al., 2018). Images were annotated by two different individuals and cross-validated to ensure that there was no substantial difference between them (Fig. S3). Trials where data were not traceable were excluded. Each image was quadrant folded to center and average intensities across axes of symmetry, and annotated for meridional and equatorial intensity peaks. We used versions of Musclex prior to version 1.14.12. These have an error in the image centering algorithm that results in the image center being rounded to the nearest pixel. This means there is a center placement error of <0.5 pixels that could result in a maximum radial compression of the image of 0.5 pixels. An improvement in centering is being implemented in future versions which reduces the error inherent in interpreting physically continuous data recorded in a pixel framework by remapping intensity based on the calculated image center.

**Cross-bridge recruitment**

Cross-bridge recruitment can be inferred by the shifting of mass away from the thick filaments and towards the thin filaments. As the flight muscle of *M. sexta* have a packing ratio of 1:3 thick to thin filaments, the intensity of the 1.0 peak contains thick and thin filament mass, while the 2.0 peak is the sum of the second-order

---

**Fig. 2. Time-resolved lattice kinematics are consistent within a trial but vary between trials.** (A) Simultaneous EMG and X-ray diffraction traces. The muscle’s activation recorded in the EMG signal is indicated by a vertical red line. The time of the EMG peak was rounded to the nearest 1/200th of a second to match the frame rate of the detector, leading to a slight mismatch with the EMG recording which had a resolution of 25,000 Hz (see Materials and Methods). In the EMG trace shown, the large amplitude peaks are from the activation of the DLMs, while the EMG also picked up the activation of the neighboring antagonistic muscle group, the dorsal ventral muscles (DVMs). These are seen in smaller amplitude spikes. Cross-bridge recruitment was inferred by the radial shift of mass between the 1.0 and 2.0 planes as cross-bridges move towards the thin filaments following activation. Lattice spacing was measured using the 1.0 reflection (see the lattice geometry schematic in the left column). Thick filament stretching was inferred from changes in the spacing of the 7.3 nm meridional reflection and thin filament stretching from changes in the axial spacing of the 5.9 nm off-meridional reflection. (B) Spike-triggered averages (STAs) vary between individuals. Data were phase averaged using the muscle’s endogenous depolarization. The mean of each time point following activation is marked with a cross (×). All the data we collected are shown in Fig. S4.
harmonic of the 1,0 and the intensity due to layers containing only thin filaments (Fig. 2). As cross-bridges move towards the thin filaments, there is a decrease of the 1,0 intensity due to the shifting of mass towards the layers of thin filaments. In contrast, the 2,0 intensity stays relatively constant as there is little change in the total mass on these planes. Therefore the ratio of 2,0 to 1,0 intensity is expected to be highest during peak cross-bridge binding. This differs from vertebrate muscle, where the ratio of the 1,1 reflection and 1,0 reflection is a surrogate for cross-bridge binding. The intensities of the 1,0 and 2,0 reflections were computed using Musclex’s Equator function, which first sets a box around the equator, uses convex hull background subtraction, and finally calculates peak intensity.

**Lattice spacing**  
Lattice spacing was measured by tracking the 1,0 reflection’s centroid motion using Musclex’s Equator function and a Voigt model fitted to three peaks: 1,0, 1,1 and 2,0. These data were gathered simultaneously with cross-bridge recruitment. Notably, the Voigt model we used within the Equator function’s framework is over-determined with only two peaks, so we also tracked the 1,1 reflection intensity in the fit model. The distance of the centroid of the 1,0 equatorial reflection from the backstop correlates by Bragg’s law to the distance between layers of the filament lattice containing the thick filament.

**Filament strain**  
Thick and thin filament strains were calculated using axial filament spacing changes recorded along the meridian and in layer lines of the diffraction pattern. We used Musclex’s Diffraction Centroids function to estimate the axial spacing of the following intensity peaks: the 14.3 nm meridional, an indicator of spacing changes between layers of myosin crowns; the 7.3 nm meridional, an indicator of spacing changes in the myosin containing thick filament backbone; and the 5.9 and 5.1 nm off meridional actin layer lines, which correspond to the pitches of the left and right genetic helices. At the exposure times we used, the actin layer lines were relatively weak. The 5.9 nm layer line intermittently yielded reliable data, while the 5.1 nm layer line was not measurable for most trials. The data presented for the 5.9 nm layer line should be interpreted with due caution as without 5.1 nm data, filament twisting and stretching cannot be differentiated.

**Permutation bootstrap**  
To assess if a signal contained significant cyclic changes at the same frequency as the muscle’s activation, we used a permutation bootstrap. We took the Fourier transform of a time series signal, which returned the power of the signal across frequency space. We then compared the power of the raw signal at wing beat frequency with the power obtained for a signal composed of the original signal, but randomly shuffled. If out of 1000 permutations, fewer than 5% had a power greater than or equal to the original signal’s power at wing beat frequency, the signal was used to compute average periodic excursions. If more than 5% had a power greater than or equal to the original signal’s, we cannot evaluate what component of the data is noise, so the signal was excluded from computing excursions. Out of the 11 trials we recorded, all showed significant periodic changes in lattice spacing, nine showed significant periodic changes in cross-bridge recruitment, seven showed significant periodic thick filament stretch, six showed significant periodic changes in the 14.3 nm centroid spacing and six showed significant periodic thin filament stretch. These results correspond to the annotator’s observations that data encoded on the equator is typically much stronger, while at the exposures lengths we used data encoded along the meridian was more challenging to track.

**Spike triggered average**  
The spike-triggered average (STA) is created by phase averaging time course X-ray diffraction data using the muscle’s depolarization as the start point. One phase is defined to be the period of time between sequential membrane depolarizations (the interspike interval). Interspike intervals were denoted using Python scripts which identify EMG peaks and down-sample their time resolution to that of the X-ray data, indicating the frame during which depolarization occurred.

**Correlation**  
The correlation of signals as a function of proportion of interspike interval lag was used to determine if there was a consistent phase offset of cross-bridge recruitment and thick filament stretch, and of cross-bridge recruitment and lattice spacing change across trials. We also calculated the correlation of two signals as a function of absolute time lag (Fig. S5) and found no consistent pattern. Correlation was calculated using Numpy’s built-in correlation function.

**Gradient boosted decision tree mechanism-free model (xGBoost)**  
xGBoost is a library to create gradient boosted decision trees that can be used for regression, classification and prediction (Chen and Guestrin, 2016). Gradient boosting iteratively combines weak hypotheses (in the case of xGBoost a weak hypothesis is a decision tree that has an imperfect prediction) that follow the gradient descent of an error loss function. At each iteration, a new tree is added with the goal of fixing the error left in the model prediction from the previous iterations. The loss function can be generalized as any differentiable function. xGBoost is a particularly fast and effective implementation of the machine learning algorithm ‘gradient boosted decision trees’ that has been used to win many Kaggle competitions (Cook, 2020). We used it to create a mechanism-free prediction of lattice dilation from filament stretching and cross-bridge recruitment data. Specifically, the model used the 14.3 nm reflection’s intensity (often used as an indicator of cross-bridge recruitment in vertebrate systems), the ratio of the reflections of the 2,0 and 1,0 planes, the 7.3 nm reflection’s centroid and the 5.9 nm off meridional’s centroid. As the change of the lattice’s shape from one time step to the next depends not only on the state of these parameters, but also their time history, we extended the information available to the model by including the time history of cross-bridge recruitment and filament stretching up to 11 time steps (0.055 s) prior to the time point to be predicted.

To create the xGBoost model, factors like the number of decision trees it uses and how many decision points each tree can encompass must be specified. These characteristic features of the model are called hyperparameters. Optimizing the choice of hyperparameters can be accomplished by methods like grid search, coordinated descent or a genetic algorithm. We chose to use a genetic algorithm. In essence, a small set of models (10 in our case) are constructed with randomly selected hyperparameters; these are a first generation. The next generation is created by combining the hyperparameters of the most successful models in the parent generation, along with random ‘mutation’ to one of the hyperparameters. We used a total of seven generations and allowed the crossing of four of the parameter models. We adapted code for an implementation of the genetic algorithm for selecting hyperparameters for xGBoost that can be found on the GitHub repository ‘Hyperparameter-tuning-in-XGBoost-using-genetic-algorithm’ (see related article at https://towardsdatascience.com/hyperparameter-tuning-in-xgboost-using-genetic-algorithm-17bd
mandally correlated with increased cross-bridge binding at a
indicated by movement of the 7.3 nm meridional reflection would be
et al., 2018), we hypothesized that the stretching of the thick filament
development, passive tension development and filament activation
relationships between the various lattice kinematics within
Our kinematic data capture filament motions within a mechanically
coupled system. Here we explore whether there are consistent
interactions between the various lattice kinematics within
individuals and, additionally, whether there are patterns common to
all individuals. As thick filament strain results from active tension
development, passive tension development and filament activation
(Wakabayashi et al., 1994; Irving et al., 2011; Ma et al., 2018; Pazzesi
et al., 2018), we hypothesized that the stretching of the thick filament
indicated by movement of the 7.3 nm meridional reflection would be
maximally correlated with increased cross-bridge binding at a
relatively fixed phase offset. However, the maximum cross-
correlation between cross-bridge recruitment and thick filament
stretch shows variable timing offsets across individual trials, demonstrating a complicated relationship between these variables
(Fig. 3A). This may be partially explained by recent work which
represents that there is a non-linear relationship between tension and thick filament extension (Ma et al., 2018). Proteins similar to titin
can also contribute to sarcomere elasticity in M. sexta (Yuan et al.,
2015) with significant non-linear behavior (Trinick, 1996;
Tskhovrebova et al., 1997; Powers et al., 2018) and temperature
dependence (Bullard et al., 2006). Finally, while stretching of the
filaments indicates internal axial force, cross-bridges also exert force
radially. This means that the magnitude of the component of cross-
bridge force applied axially changes as a function of the angle
between motors and binding sites (Schoenberg, 1980; Williams et al.,
2010). In light of these considerations, it would be remarkable if the
movement of mass towards the thin filaments alone explained the
periodic stretching of the thick filaments, or the periodic dilation of the
lattice (Fig. 3B); but taken together, can we use these data to fully
model the system?

If the data are sufficient to explain one another mechanistically,
even though the linkage is non-obvious, they should be sufficient to
train a mechanism-free, data-based model (although by creating a
mechanism-free model we cannot exclude the possibility that the data
are insufficient to explain one another mechanistically, but contain
enough correlate information to successfully predict one another). We used a gradient boosting decision tree algorithm housed in the
xGBoost library (Chen and Guestrin, 2016) to train a mechanism-free
model. First we trained the model with 75% of a trial’s data, using the
other 25% as a test set to evaluate model performance. We set up the
model to predict lattice spacing from filament stretching and cross-
bridge recruitment as lattice spacing is typically the cleanest of the
signals tracked in X-ray diffraction due to its intensity. In addition to
using the current state of filament stretch and cross-bridge recruitment
to predict the current lattice spacing, we also provided the model with the
time history of the predictor variables up to 11 time steps (0.055 s)
previous to the current state. For these within-trial predictions the
random forest-based model performed well, with a root mean square
error (RMSE) of 0.27 nm, demonstrating that it is possible to predict
lattice spacing change from filament stretching and cross-bridge
recruitment within a trial (Fig. 3C).

We then addressed our larger question – can we create a
prediction of lattice spacing change from cross-bridge recruitment
and filament stretching data that holds across individuals? We
iteratively withheld the data from one individual as a test case and
trained the model with the data from all other individuals. The
average RMSE across all trials was 0.78 nm. This shows that the
themes the model uses to create intra-individual predictions do not
generalize well across individuals (Fig. 3D).

**DISCUSSION**

Combining high-speed, time-resolved X-ray diffraction with
simultaneous recording of the electrical activation of the synchronous
flight muscles of the hawkmoth M. sexta, we resolved myofilament
lattice kinematics during fully intact tethered flight. Taken together, our
data reveal intra-individual patterns of axial myofilament stretching,
radial spacing changes and cross-bridge recruitment that follow the
dependent activation of muscles. This method gives us a window into
the molecular motions that underlie muscle force production.

**Cross-bridge recruitment**

Although X-ray evidence for the timing of the excitation–
contraction pathway has been used in isolated fibers (Reconditi
2e581b17). In the interest of reproducibility, the model’s
hyperparameters can be found in Table S1.

**RESULTS**

We recorded cross-bridge recruitment, axial filament strains and
radial lattice dilation as a function of endogenous muscle activation
during in vivo tethered flight. These were indicated, respectively, by
the ratio of the intensities of the 2.0 to the 1.0 equatorial reflections;
axial shifts of the centroid of the 7.3 nm meridional reflection and
axial shifts of the 5.9 nm actin off meridional reflection, and finally
radial shifts in the position of the equatorial reflections (Fig. 2A).
We also recorded the axial shifting of the 14.3 nm meridional
reflection. With a detector frame rate of 200 Hz and wing beat
frequencies ranging between 13 and 19 Hz (mean 16, s.d. 2), we
obtained 11–15 X-ray diffraction images from each cycle of
shortening and lengthening. Each trial lasted 1 s, and we created
STAs by phase averaging the data based on the peaks of each
individual’s EMG. Most individuals had consistent STAs; however,
there was variability between individuals in the time course and
excursions of their STAs for a given data type (Fig. 2; Fig. S4). We
calculated the excursion of a signal as the difference between the
maximum and minimum of the STA for each trial.

**The timing and extent of cross-bridge recruitment, filament
stretching and lattice dilation are revealed in STAs**

By using a synchronous muscle group, we were able to correlate
muscle activation and the resulting recruitment of myosin molecular
motors to the thin filaments. The peak cross-bridge recruitment
occurred an average of 0.024 s (s.d. 0.026) following activation,
with a resolution of 0.005 s. We measured the extent of filament
stretching by calculating the excursion (maximum minus minimum)
of the 7.3 nm reflection’s STA. This revealed that thick filaments
stretched by an average 0.75% (s.d. 0.32) across seven trials. By first
calculating the excursion of each trial and subsequently computing
the means across trials we avoid the blunting of the signal that can
occur if the excursion is calculated from the amalgamation of all
trial’s normalized STAs. Likewise, we calculated the lattice’s
dilation as the maximum minus the minimum of the STA for each
trial, with a mean taken across all 11 trials. The lattice diluted by
2.72% (s.d. 1.47), which corresponds to 1.24 nm (s.d. 0.66).

For each of these data types, the pattern of the STA was relatively
consistent across many cycles of shortening and lengthening within
an individual trial. This is especially apparent in the case of the
lattice spacing (the d1,0), which, as the clearest diffraction signal, is
least subject to error during annotation. However, the STAs reveal
extensive variation across individuals, which means that there is a
large standard deviation in the timing and extent of myofilament
lattice kinematics.

**Inter-relationship of lattice kinematics: inter- and
intra-individual patterns**

Our kinematic data capture filament motions within a mechanically
coupled system. Here we explore whether there are consistent
relationships between the various lattice kinematics within
individuals and, additionally, whether there are patterns common to
all individuals. As thick filament strain results from active tension
development, passive tension development and filament activation
(Wakabayashi et al., 1994; Irving et al., 2011; Ma et al., 2018; Pazzesi
et al., 2018), we hypothesized that the stretching of the thick filament
indicated by movement of the 7.3 nm meridional reflection would be
maximally correlated with increased cross-bridge binding at a
relatively fixed phase offset. However, the maximum cross-

The variation in lattice kinematics that we recorded in a fully intact system could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables. For example, temperature varies both spatially and temporally in the intact system and could arise from several latent variables.

Temperature gradients correlate with variation in lattice kinematics at the molecular scale, and molecular-scale variation corresponds to functional gradients across the muscle group at the organism scale (George et al., 2013). In addition to gradients across a muscle group, the mean temperature of the muscle group increases with increasing wing beat frequency in M. sexta (Heinrich and Bartholomew, 1971). Temperature may contribute to the observed variation in our system as the diffusion of substrates like calcium ions and molecular motors is slower in cool muscle. However, the experimental constraints of simultaneous EMG recordings and high-speed, time-resolved X-ray diffraction in a naturally functioning animal limit our ability to resolve spatiotemporal patterns of temperature in the muscle. In favor of an intact preparation, we did not control temperature and all of the moths were flapping their wings at different frequencies of their own volition. Although temperature probably contributes to the variation we observe in our data, diversity in the timing of maximum cross-bridge recruitment could also arise from other mechanisms, such as other steps in the excitation–contraction pathway, or co-occurring lattice spacing changes and filament stretching.
Although the ratio of the intensities of the 2.0 and 1.0 reflections is often used to quantify the shifting of mass between them (Irving, 2006), peak intensities could in theory be affected by changes in lattice ordering (Bershitsky et al., 2009) over a cycle of shortening and lengthening. Additionally, shifting of mass from the thick to the thin filaments does not necessarily imply binding. However, despite these caveats, the shifting of mass is well correlated with strong motor binding in vertebrate muscle (Squire, 1997; Harford and Squire, 1992). This ratio is an accessible surrogate for cross-bridge recruitment during time-resolved X-ray diffraction.

Filament stretching
Filament stretching modulates a muscle’s function. Axial filament stretching accounts for a large component of the sarcomere’s total compliance (about 70%; Wakabayashi et al., 1994), enabling the return of stored elastic strain energy. The stretching of filaments also means that cross-bridges do not act independently of one another. Instead, as the relative separation between binding sites and cross-bridges changes, binding probability is also altered. Spatially explicit modeling demonstrates that these changes in axial register may mediate force output (Daniel et al., 1998). In addition to changes in axial register, strain in the thick filament may alter its twist. In Lethocerus, asynchronous flight muscle thick filament strain is accompanied by a change in twist of about 12 deg that would help move heads closer to actin target zones as thick and thin filaments are strained (Perz-Edwards et al., 2011). Unfortunately X-ray patterns from the flight muscle of M. sexta lack the rich layer lines that Lethocerus muscle displays, so we could not identify changes in helical twist of the thick filament. The extent to which filament twist occurs in M. sexta as a function of filament extension remains conjectural until other experiments are devised to detect such changes.

Despite its functional significance, the extent of filament stretch occurring during natural muscle function was unknown until recent evidence from time-resolved X-ray diffraction revealed subtle (0.2%) thick filament stretching in the dominant flight muscles of Drosophila (Dickinson et al., 2005). Our measurements show that the thick filament stretches by an average of 0.75% (s.d. 0.32) across seven trials, as indicated by changes in the 7.3 nm meridional intensity peak (Fig. 2). While the symmetry of the thick filament of M. sexta is not yet known it may be different from vertebrate filaments, possibly akin to Lethocerus, which has 4-fold rotational symmetry (Reedy et al., 2000; Hu et al., 2016). Thus the 7.3 nm reflection may not be exactly equivalent to the M6 reflection in vertebrate muscle, nor the 14.3 nm reflection to the M3. With this caveat, in analogy with vertebrate muscle, the 7.3 nm reflection is likely to include contributions from the second order of the 14.3 nm myosin head repeat with additional contributions from the thick filament backbone (Brunello et al., 2006). The imperfect correlation of the spacing and intensity changes of the 7.3 nm reflection and the 14.3 nm reflection in vertebrate muscle, however, indicates that the 7.3 nm reflection is dominated by structures in the backbone; meanwhile, the 14.3 nm reflection is dominated by the periodicity of the myosin heads so that spacing changes in the 7.3 nm reflection may be used as a measure of thick filament extensibility (Huxley et al., 1998; Linari et al., 2000; Brunello et al., 2006; Ma et al., 2018). We have shown the cross-correlation of the 7.3 nm and 14.3 nm repeats from these data in the Supplementary information (Fig. S1). We conjecture that the same pattern seen in vertebrate muscle holds for the flight muscle of M. sexta. Although larger than those observed in Drosophila, the values we report are near those observed in isolated vertebrate fibers under isometric contraction (Huxley et al., 1994; Wakabayashi et al., 1994; Brunello et al., 2006). This result confirms the relevance of these parameters to in vivo function in a synchronous muscle group during natural function. Additionally, by measuring the extension of filaments within individuals rather than averaging across trials, the blunting of the signal that occurs when averaging phase offset data is avoided.

Lattice dilation
Lattice function is also impacted by the spacing of the myofilament lattice, which is thought to play a role in the Frank–Starling mechanism in the mammalian heart (Moss and Fitzsimons, 2002). Cross-bridge binding alters lattice spacing, and in turn their binding probability and the direction of the forces they generate are regulated by lattice spacing (Schoenberg, 1980). Radial cross-bridge extension has also been proposed as a site of elastic energy storage that could be returned to power cyclic contraction (Williams et al., 2012; George et al., 2013). Through mechanisms such as these, lattice spacing change results in a steeper length–tension curve than would be produced if only filament overlap changed during contraction (Williams et al., 2010). As these muscles act on the steep ascending portion of their length–tension curve, they generate larger forces in response to perturbations that stretch them, without a need to modify nervous control. Therefore in the case of cardiac muscle when blood pressure suddenly rises and increases ventricular filling, or in the case of flight muscle when a gust of windbuffets against the wing, the muscle autonomously contracts more forcefully (Tu and Daniel, 2004). This is an example of a cell-scale set point that yields a reflexive response to environmental perturbations at the organism scale, lending robustness to rapidly changing external demands (Moss and Fitzsimons, 2002).

Isolated muscle preparations have yielded mixed interpretations of how lattice spacing changes over the course of a contraction, although it is clear that lattice spacing is influenced by both mechanical and electrostatic interactions (Smith, 2014). Often the lattice is assumed to have either a constant spacing during contraction, or to be isovolumetric. However, these assumptions do not capture the extent of lattice spacing change observed experimentally. For instance, in skinned fiber preparations it was shown that during force generation lattice spacing tended towards a spacing near that observed when the membrane was intact (Millman, 1998). Meanwhile it was shown in relaxed, intact vertebrate muscle fibers that the sarcomere can be approximated as being isovolumetric, but when cross-bridges are active both axial tension and sarcomere length are determinants of lattice spacing (Bagni et al., 1994). Are lattice spacing changes occurring during in vivo muscle function?

The average lattice spacing excursion we measured across 11 trials was 2.72% (s.d. 1.47), corresponding to 1.24 nm (s.d. 0.66). These data stand in contrast to the results of Irving and Maughan (2000), which showed no measurable lattice spacing change in the flight muscles of Drosophila to a resolution of 0.05 nm (the equivalent of ±0.1% lattice spacing change in their system). While these are significant changes in spacing, they are smaller than the approximately 4% change in spacing needed to maintain a constant volume based upon the strains of this muscle group (about 9%; Tu and Daniel, 2004). The average lattice spacing change we measured corresponds to an alteration in the force generated in osmotically compressed demembranated myofibrils of nearly 20% (Williams et al., 2013). Therefore we expect that lattice spacing change may be an important determinant of the force produced by this muscle group. Moreover, the maximum lattice spacing excursion we recorded was nearly twice the mean (2.5 nm). Akin to cross-bridge
recruitment and filament stretching, lattice spacing change followed a stereotyped time course within individual trials, but the time course of lattice dilation demonstrated great breadth across trials. Similar parameters to those noted for cross-bridge recruitment and filament stretch may also give rise to the variation we observed in lattice spacing.

Inter-relationship of lattice kinematics: inter- and intra-individual patterns

Ultimately, the myofilament lattice is a mechanically coupled system, but we do not know how each of the pieces relate to one another across the widely variable STAs that we documented. Moreover, cross-correlation did not reveal thematic phase relationships between kinematics across trials, but rather highlighted the variation in the timing of kinematics relative to each other.

In response to these limitations, we built a mechanism-free machine-learning model to predict lattice dilation using the other kinematics we recorded. While the model performed well within trials, it was not able to forecast across trials effectively. The model cannot find relationships in the training set that explain the test set data, and instead of interpolating the model must extrapolate to forecast across trials, reflecting the visibly variable STAs. This may indicate that there are mechanisms that we did not record which account for the inter-trial variation. Latent variables like temperature, externally applied forces, the timing of activation and antagonistic muscle activation are components that may be necessary to mechanistically explain myofilament lattice dynamics. These results demonstrate that the myofilament lattice uses a panoply of kinematic combinations, the breadth and significance of which we have yet to grapple with during natural function.

Conclusion

At the organism scale muscle exhibits diverse functionality, in turns powering motion, stabilizing the body, storing energy and dissipating energy (Dickinson et al., 2000). Molecular to organism scale feedback enables muscle to meet these demands. We recorded myofilament lattice kinematics during natural behavior at all scales, revealing that thick filaments stretch by 0.75% (s.d. 0.32) and that the lattice dilates by 2.72% (s.d. 1.47). By using the synchronous flight muscles of M. sexta, we were able to record that peak cross-bridge recruitment occurs 24 ms (s.d. 26) after activation in vivo. Despite the inherent uncertainty in interpreting X-ray diffraction data from a muscle group with unresolved ultrastructure, this system has promise for understanding the in vivo dynamics of a muscle group with striking similarities to human cardiac muscle. We recorded extensive inter-individual variation in the timing and extent of lattice kinematics that could not be predicted by a powerful, mechanism-free model. The machine learning model we used capitalizes on weak patterns in data to make predictions, regardless of whether those patterns are mechanistic or correlative relationships. The inability of this model to forecast across trials indicates that latent variables (such as variation in the timing of muscle activation and the interaction of many muscles in the animal’s body) may give rise to the behavior we documented. While we cannot pinpoint the source of the variation we observed, it points to the need to explore how muscle uses a broad palette of kinematics to produce functional movement in a constantly changing environment.

Acknowledgements

The authors gratefully acknowledge the thoughtful feedback of Joseph Powers, Abigail von Hagel and Michael Regnier on muscle biophysics; and the feedback of Valentina Staneva, Callin Switzer, Satpreet Singh and Bigni Brunton on data analysis. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the Pilatus 3X1M detector was provided by grant 15100D18090-01 from the National Institutes of Health.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

This project was supported by the National Institutes of Health (P41 GM103622), the Army Research Office (W911NF-14-1-0396 to T.L.D.) and the Joan and Richard Komen Endowed Chair to T.L.D. S.A.M. was supported by a Bioengineering Cardiac Training Grant from the National Institute of Biomedical Imaging and Bioengineering (T32EB1650) and a fellowship from the ARCS Foundation. A.M.A. was supported by NIH P03 AR074990. The content is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health. Deposited in PMC for release after 12 months.

Data availability

All code used can be found in the GitHub repository: In_vivo_Myofilament_Lattice_Kinematics. All the data that we used in preparing the manuscript can be found in the Dryad Digital Repository (Malingen et al., 2020): dryad.1sm8p5k1

Supplementary information

Supplementary information available online at https://jeb.biologists.org/lookup/doi/10.1242/jeb.224188.supplemental

References


8


Malingen, S. A., Asenjo, A. M., Cass, J. A., Ma, W., Irving, T. C. and Daniel, T. L. (2020). Data from In vivo X-ray diffraction and simultaneous EMG reveal the time course of myofilament lattice dilation and filament stretch. Dryad Data https://doi.org/10.5061/dryad.1m9jk51


Supplement

Fig. S1. The maximum correlation between the 7.3 nm repeat and 14.3 nm repeat occurs at small but variable phase offsets across trials. The signals are minimally correlated at a phase offset midway through the interspike interval (the duration of time between one muscle activation and the next). The M6 and M3 may record different structures, explaining why signals are not perfectly phase locked. The reason why this is the case follows. X-ray diffraction takes a Fourier transform of the myofilament lattice. In short exposure images the intensities recorded by the detector due to periodic structures are hard to disambiguate from noise. By averaging many images strong traces from periodic structures emerge. Frame averaging is analogous to increasing the total photon count. Similarly, reflections close to the backstop are the composite of larger numbers of photons. Signal averaging is more important when there is a low signal to noise ratio. For labile structures like cross-bridges, a large photon count is needed to create a crisp signal that rises above the background noise. In contrast the more stable thick filament backbone’s periodicity is less dependent on averaging. Hence, the intensity due to the myosin crown repeat predominates close to the backstop, but as the distance from the backstop increases, the signals from labile structures will become more diffuse, while those from stable structures retain their integrity. Therefore the M6 and M3 may record different structures, explaining why signals may not be perfectly phase-locked.

Fig. S2. A single, x-ray diffraction image from Moth 15 trial 1. Greater clarity could have been gained from longer exposures at the expense of reduced time resolution. Since the conversion from pixel to nanometer space by Bragg’s law is non-linear, error disproportionately effects data recorded close to the backstop. An error of one pixel corresponds to 0.04 nm for the 7.3 nm spacing, while for the 5.9 actin off meridional an error of one pixel corresponds to an error of 0.03 nm. Meanwhile for the 14.3 nm reflection and the lattice spacing, which are both closer to the center of the image, an error of one pixel corresponds to 0.17 nm and 1.68 nm respectively.
Fig. S3. Quantification of inter-annotator error. To verify that there was not significant inter-annotator variation we compared their pixel space annotations for a time series (a stack of images). For the d1.0 data we also compared a version of Musclex with an auto-orienting patch to Musclex. To quantify the variation between annotators shown in the time series plots on the left hand side, we computed a Root Mean Square Goodness of Fit (RMS GOF) between the two annotated image stacks. This value was compared to the distribution of RMS GOFs for 10,000 random permutations of the time series. We performed this for 4 trials with 15 time points from the d1.0 centroid, the 7.3 nm centroid and the 14.3 nm centroid. Two of the 4 cross validated trials weren’t used in the final analysis; in one case because the muscle activation couldn’t be determined from the EMG, and in the other case because the moth quit flying in the middle of the trial. The right hand plots show the distribution of RMS values calculated from the permutation bootstrap, with vertical lines denoting the RMS value for each annotation comparison. The annotator-comparison RMS GOF values are outside of the permutation bootstrap distribution, suggesting that the results are not explained by chance. The low annotator-comparison RMS GOF values indicate that the variation between the annotators was low. In addition to the RMS GOF, we calculated a p-value for each trial. The p-value addresses the null hypothesis that the order of the data does not affect the RMS GOF value, with a low p-value indicating that the annotations are consistent in their placement within the series. A low RMS GOF value indicates low disparity in annotator recorded values. The distributions for our data were typically unimodal. The average RMS of the d1.0 centroid was 0.07, and the average p value was 0.0008. The average RMS of the 7.3 nm was 0.70 and the average p value was 0.02. The average RMS of the 14.3 nm was 0.41, while the p value was 0.03.
Fig. S4. All of the data we collected is shown in this panel of STAs.
Fig. S5. The cross-correlation between a pair of signals given an offset of the signals measured in seconds shows inconsistent phase relationships across trials. This is in contrast to the other plots of cross correlation as a function of proportion of interspike interval. In none of these cases does a given offset in seconds yield the maximum correlation across trials.

Table S1. The optimal hyperparameters determined by the genetic algorithm for each trial are listed. The white cells are those that were used for the case where the model was trained on 75% of the data and tested on the remaining 25% of the data within a trial. The gray cells are the parameters that were used for the case where the model was trained on all trials except for the withheld trial upon which it was tested.

<table>
<thead>
<tr>
<th>Trial</th>
<th>learningrate</th>
<th>nestimators</th>
<th>maxdepth</th>
<th>minchildweight</th>
<th>gamma</th>
<th>subsample</th>
<th>colsamplebytree</th>
</tr>
</thead>
<tbody>
<tr>
<td>m07_t01_15</td>
<td>0.16</td>
<td>241.0</td>
<td>7.0</td>
<td>5.1</td>
<td>0.04</td>
<td>0.77</td>
<td>0.79</td>
</tr>
<tr>
<td>m07_t03_15</td>
<td>0.26</td>
<td>98.0</td>
<td>1.0</td>
<td>1.53</td>
<td>1.19</td>
<td>0.29</td>
<td>0.64</td>
</tr>
<tr>
<td>m07_t06_15</td>
<td>0.05</td>
<td>135.0</td>
<td>3.0</td>
<td>0.72</td>
<td>1.22</td>
<td>0.62</td>
<td>0.34</td>
</tr>
<tr>
<td>m10_t02_16</td>
<td>0.9</td>
<td>134.0</td>
<td>2.0</td>
<td>2.1</td>
<td>1.81</td>
<td>0.55</td>
<td>0.27</td>
</tr>
<tr>
<td>m11_t02_16</td>
<td>0.45</td>
<td>111.0</td>
<td>9.0</td>
<td>7.38</td>
<td>2.37</td>
<td>0.59</td>
<td>0.59</td>
</tr>
<tr>
<td>m11_t04_16</td>
<td>0.44</td>
<td>204.0</td>
<td>2.0</td>
<td>5.96</td>
<td>0.89</td>
<td>0.8</td>
<td>0.75</td>
</tr>
<tr>
<td>m12_t02_16</td>
<td>0.43</td>
<td>98.0</td>
<td>1.0</td>
<td>7.18</td>
<td>0.79</td>
<td>0.21</td>
<td>0.42</td>
</tr>
<tr>
<td>m14_t05_16</td>
<td>1.0</td>
<td>160.0</td>
<td>4.0</td>
<td>10.0</td>
<td>7.85</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>m14_t03_16</td>
<td>0.22</td>
<td>248.0</td>
<td>8.0</td>
<td>0.16</td>
<td>0.51</td>
<td>0.62</td>
<td>0.48</td>
</tr>
<tr>
<td>m15_t01_16</td>
<td>0.29</td>
<td>30.0</td>
<td>7.0</td>
<td>6.8</td>
<td>0.73</td>
<td>0.82</td>
<td>0.43</td>
</tr>
<tr>
<td>m15_t03_16</td>
<td>0.34</td>
<td>48.0</td>
<td>2.0</td>
<td>6.68</td>
<td>0.01</td>
<td>0.93</td>
<td>0.23</td>
</tr>
<tr>
<td>m07_t01_15</td>
<td>0.31</td>
<td>198.0</td>
<td>5.0</td>
<td>10.0</td>
<td>7.95</td>
<td>0.85</td>
<td>0.91</td>
</tr>
<tr>
<td>m07_t03_15</td>
<td>0.11</td>
<td>10.0</td>
<td>4.0</td>
<td>10.0</td>
<td>0.61</td>
<td>0.63</td>
<td>0.92</td>
</tr>
<tr>
<td>m07_t06_15</td>
<td>0.05</td>
<td>26.0</td>
<td>3.0</td>
<td>6.69</td>
<td>7.42</td>
<td>0.76</td>
<td>0.13</td>
</tr>
<tr>
<td>m10_t02_16</td>
<td>0.21</td>
<td>54.0</td>
<td>4.0</td>
<td>5.72</td>
<td>4.68</td>
<td>0.67</td>
<td>0.79</td>
</tr>
<tr>
<td>m11_t02_16</td>
<td>0.06</td>
<td>217.0</td>
<td>1.0</td>
<td>9.41</td>
<td>7.24</td>
<td>1.0</td>
<td>0.36</td>
</tr>
<tr>
<td>m11_t04_16</td>
<td>0.35</td>
<td>30.0</td>
<td>4.0</td>
<td>9.66</td>
<td>9.13</td>
<td>0.74</td>
<td>0.9</td>
</tr>
<tr>
<td>m12_t02_16</td>
<td>0.07</td>
<td>19.0</td>
<td>6.0</td>
<td>6.37</td>
<td>5.45</td>
<td>0.77</td>
<td>0.4</td>
</tr>
<tr>
<td>m14_t05_16</td>
<td>0.35</td>
<td>319.0</td>
<td>7.0</td>
<td>10.0</td>
<td>7.39</td>
<td>0.62</td>
<td>0.49</td>
</tr>
<tr>
<td>m14_t03_16</td>
<td>0.59</td>
<td>55.0</td>
<td>7.0</td>
<td>5.18</td>
<td>2.58</td>
<td>0.99</td>
<td>0.88</td>
</tr>
<tr>
<td>m15_t01_16</td>
<td>0.09</td>
<td>136.0</td>
<td>5.0</td>
<td>10.0</td>
<td>8.6</td>
<td>0.21</td>
<td>0.52</td>
</tr>
<tr>
<td>m15_t03_16</td>
<td>0.25</td>
<td>179.0</td>
<td>10.0</td>
<td>10.0</td>
<td>6.04</td>
<td>0.12</td>
<td>0.1</td>
</tr>
</tbody>
</table>